PB84-174382

Effects of Holding Time and Temperature on Coliform Numbers in Drinking Water

(U.S.) Environmental Monitoring and Support Lab.
Cincinnati, OH.

1983

U.S. Department of Commerce
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(Please read Instructions on the reverse before completir

1. REPORT NO.	2.	3. R PB84-174382
EPA-600/J-83-153		

4. TITLE AND SUBTITLE	5. REPORT DATE
Effects of Holding Time and Temperature on Coliform Numbers in Drinking Water (Journal Version)	1983
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
A. E. McDaniels and R. H. Bordner	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Environmental Monitoring and Support Laboratory U.S. EPA - Cincinnati 26 W. St. Clair St. Cincinnati, OH 45268	11. CONTRACT/GRANT NO. N.A.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Environmental Monitoring and Support Laboratory U.S. EPA - Cincinnati 26 W. St. Clair St. Cincinnati, OH 45268	General Review 14. SPONSORING AGENCY CODE EPA/600/06

15. SUPPLEMENTARY NOTES
REF: Journal AWWA, 75(9):458-463, 1983

16. ABSTRACT

Natural coliform populations in finished drinking water samples held at 5 and 22°C declined significantly after 24, 30 and 48 h as measured by the membrane filter (MF) and multiple tube fermentation, most probable number (MPN) methods. Twelve samples held for 24 h at 5°C and 22°C lost 34% and 87% respectively, of the initial MF count. Initial unacceptable counts ranging from 31 to 5/100 mL for 5 samples (42%) declined to acceptable levels of 4 or less/100 mL by 24 h. The most significant effect on coliform rates of decline came from holding temperature. The rate of decline was 4.5 times greater at 22°C than at 5°C. Regression analysis indicated that for samples held at 5°C the rate of decline increased with high pH values and increased progressively over time, measured in days from the start of the study. This study indicated that samples with initial coliform counts as high as 100/100 mL could have been reported as satisfactory. For the waters examined, a holding time of 30 h at ambient laboratory temperature, as currently specified in drinking water regulations, would have resulted in inaccurate and misleading information on the coliform counts.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Drinking Water Microbiological Methods MF Method Standard Plate Count Coliform Citrobacter freundii	Water Microbiology Total Coliform Holding Time Holding Temperature	

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	7
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

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RESEARCH AND TECHNOLOGY

EPA-600/J-83-153
JOURNAL ARTICLE

Effects of holding time and temperature on coliform numbers in drinking water

Audrey E. McDaniels and Robert H. Bordner

Concern has often been expressed about the reliability of coliform counts from drinking water samples held in transit before analysis. Samples collected from a municipal distribution system were split, stored at ambient (22° C) and refrigerator (5° C) temperatures, and analyzed over time for total coliform and heterotrophic bacterial densities. Coliform populations declined significantly at both temperatures after 24 hours. The rate of decline was 2.5 orders of magnitude greater at 22° C than at 5° C. Average losses in 24 hours were 34 percent at 5° C and 87 percent at 22° C. Growth and interference of heterotrophic bacteria with coliform colonies on membrane filter plates were frequently observed for samples held at ambient temperature for 30 hours. A model based on actual coliform recovery was developed to predict initial coliform counts in samples held at various times before examination. For the waters examined, samples held at 22° C for 30 hours with initial coliform counts as high as 100/100 mL could have been reported as satisfactory, thus resulting in inaccurate and misleading information.

The effects of holding time and temperature on microbiological analysis of drinking water remain undetermined after nearly 100 years. Concern for this problem can be traced back to 1899 when Jordan and Irons[1] stated: "all experienced water analysts insist upon the desirability of plating a sample of water immediately after collection." It is generally recognized that water samples collected for microbiological analyses must be examined as soon as possible because of changes that could occur in the bacterial densities owing to the chemical and physical characteristics of the sample and interaction of the biota.

Many investigations have been reported, but basic differences in the conditions of the studies make comparisons difficult, if not impossible. Jordan[2] observed a gradual decrease in bacterial densities of polluted waters held at either ambient or refrigerator temperatures. Caldwell and Parr[3] compared coliform recovery from iced and ambient well water samples held for various time periods and reported losses of 40–50 percent within a few hours at both temperatures. The Public Health Laboratory Service Water Subcommittee of Great Britain[4] reported that storage for 6 hours at ambient temperature was not always satisfactory, and, by their standards, some samples showed significant changes after 6 hours in the refrigerator. Geldreich et al[5] found the mean coliform density at 24 hours was 72 percent of the mean at 2 hours for 18 samples collected from wells, lakes, and rivers and held at 5°C. On the other hand, Lonsane et al[6] examined marginally polluted waters held at ambient (22°C–39.5°C) and refrigerator temperatures (0°C–10°C) and reported that membrane filter (MF) counts of coliforms from samples held up to 48 hours were not significantly different from those found initially. However, a table of geometric means of MF counts indicated a 20 percent loss at refrigerator temperatures in 24 hours and a 27 percent loss at ambient temperatures in 48 hours. Standridge and Lesar[7] examined 28 samples of heavily polluted water with initial coliform counts that ranged between 10^2/mL and 10^6/mL and found little change after storage at 2°C–4°C for 24 hours. Dutka and El-Shaarawi[8] stored waters with various pollution loads at 1.5°C and reported that at least 75 percent of the samples had a constant level of bacteria for 24 hours, but there was little evidence that microbial populations were stable over 48 hours. The major problems in interpreting data from earlier studies, in terms of the effects of holding time and temperature on drinking water samples, are that most of the samples had high bacterial counts rather than the relatively low counts found in drinking waters and that most results were based on the multiple-tube fermentation method, with results reported in terms of the most probable number (MPN) rather than the more precise MF method.

To keep changes in bacterial densities to a minimum and to provide more valid results, *Standard Methods for the Examination of Water and Wastewater*[9] and *Microbiological Methods for Monitoring the Environment*[10] recommend polluted samples be held at temperatures less than 10°C and from 1°C–4°C, respectively, and be analyzed within 6–8 hours of collection. However, these methods permit drinking water samples that are not delivered to the laboratory by the collector to be held in transit at ambient temperatures for up to 30 hours before analysis. The rationale is that changes are less likely to occur in drinking water samples because they are usually of good quality and have low bacterial densities. Though some state laboratories analyze drinking water samples that have been held as long as 48 hours, they are required to indicate that the data may be invalid. Many laboratories, particularly those in sparsely populated states where sampling sites are distant from the laboratories and rapid transportation is not available, have objected to the difficulty and expense of adhering to the 30-hour holding limit for drinking water samples.

The purpose of this study was to investigate sample-holding effects by using current methodology and to supply more reliable data for consideration in future revisions in drinking water regulations. This investigation was based on (1) coliform-positive samples from a drinking water distribution system rather than from polluted ambient waters, (2) direct coliform counts by the MF technique rather than the MPN index, and (3) an experimental design that provided a low within-sample variance and produced data that conformed to currently used statistical evaluations.

Materials and methods

Sampling site. The sample source was a public water supply from a municipally operated system in the Midwest. Samples were collected from a fire hydrant located

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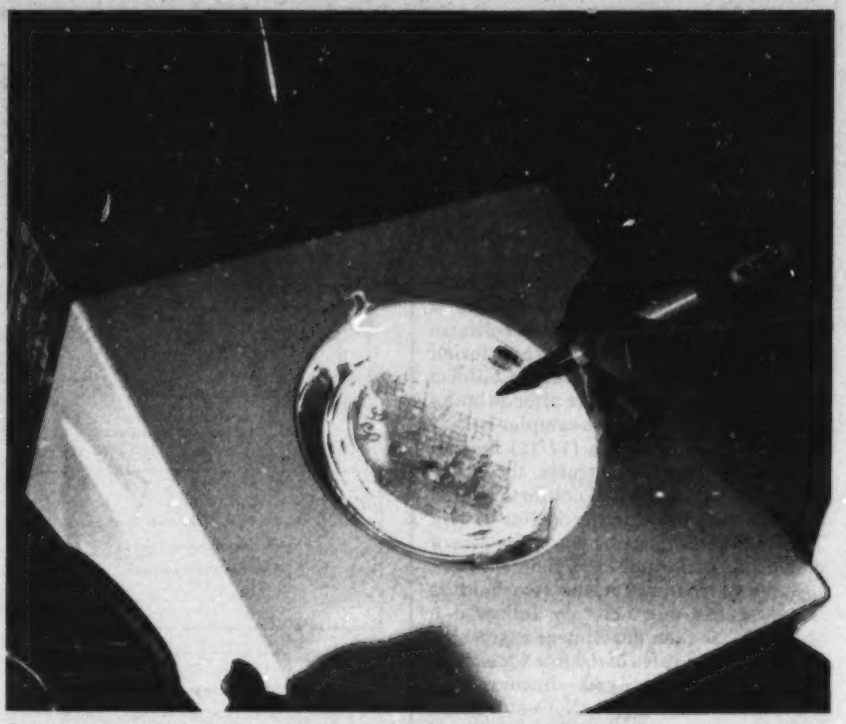
near the end of a main, where flow was at a minimum and the potential for the occurrence of coliforms was high.

Sample collection and processing. Samples were collected weekly or biweekly from Sept. 8 to Dec. 31, 1981. Water was allowed to flow for approximately 2 to 3 min (until clear) before samples were collected. Figure 1 illustrates the experimental design used to produce valid data and to reduce variability of sample measurements. A sterile polypropylene bottle with a capacity of 50 L was filled, and the sample was thoroughly mixed. One-litre aliquots were aseptically removed from the 50-L samples at the test site for chemical and physical analysis. For chlorine neutralization, sufficient sodium thiosulfate was added to the remaining 49 L to provide a final concentration of 100 mg/L. Initial temperature, pH, and free and total chlorine were measured in the field.

Samples were transported to the laboratory for analysis within 1 hour of collection. Chlorine was measured with a commercially available kit.* Turbidity and specific conductivity were measured with a turbidimeter† and a specific conductivity instrument.‡ The 49-L samples were shaken by hand for 5 min, and six subsamples were dispensed aseptically into 4-L sterile polypropylene bottles. Three subsamples were stored in the dark at 5°C, and three were stored at 22°C. Aliquots of 500 to 700 mL were poured aseptically from each thoroughly mixed subsample immediately and at holding times of 2, 6 or 12, 24, 30, and 48 hours for microbiological analysis. The 2-and 6-hour analyses were completed on only the first four samples because results indicated that these data were not required to determine the rate of coliform decline.

Microbiological analyses. The initial microbiological examination began in the laboratory within 1 hour of collection. Aerobic and facultative anaerobic heterotrophic bacteria populations were enumerated by using the standard plate count (SPC) method.[9] Total coliform (TC) counts were obtained by using the MPN procedure with lauryl tryptose broth§ (LTB) presumptive medium, confirmed in brilliant green lactose bile broth§ (BGLB), and by using the MF technique with m-Endo§ agar.[9]

All subsamples were examined by the MF technique. Results from preliminary examinations of the water source were used in selecting sample volumes that would provide acceptable coliform counts (≤80) by the MF technique. Three incremental volumes per subsample were filtered. The volumes used depended on the number of coliforms anticipated, the degree of turbidity that may have interfered with filtration, and the expected background population capable of growth on the medium. A minimum of two replicate plates were prepared for each volume.



Colonies of aerobic and facultative anaerobic heterotrophic bacteria that grew on standard plate count agar are counted.

Twenty coliform colonies were randomly selected for verification from initial plates for each sample. If fewer than 20 colonies were found, all coliform colonies were verified.[10]

One subsample from each holding time and temperature was analyzed by the SPC and MPN methods. Sample dilutions for SPC ranged from 1 mL to 1×10^{-5} mL, and each dilution was analyzed in triplicate. A five-tube series of three dilutions was used for MPN analyses. The dilutions prepared ranged from 10 mL to 1×10^{-5} mL as needed. Five percent of the MPN analyses were carried to completion.[10]

A commercially available index** was used to identify coliform isolates. From three to five metallic sheen colonies per sample were processed through this index.

Statistical analysis. The statistical tests used for examination of the data included scattergraphs, *F*-statistic, r^2, Pearson product moment correlations, paired *t*-test, analysis of variance for repeated measures, regression analysis, and maximum likelihood estimates (MLE).[11,12] Total coliform and SPC mean sample counts were transformed to common logarithms ($\log_{10}$) to convert the data to a normal distribution for analysis.

Scattergraphs, *F*-statistic, and r^2 results indicated the suitability of a linear regression model for evaluating the MF data. Regression and MLE analyses were used to determine possible significant effects of seasonal changes (expressed as days of sample collection from the beginning to the end of the study), initial temperature, initial pH, free and total chlorine, turbidity, and specific conductivity on the rates of decline in MF coliform counts. The same set of variables was also examined for effects on $\log_{10}(N_t/N_0)$ rates for SPC at 24, 30, and 48 hours, where N_t represents the mean count/100 mL after time t, and N_0 is the initial mean count/100 mL.

The MLE rates of decline for MF coliforms and the 95 percent confidence intervals were used to predict initial counts for samples held 6, 12, 24, or 30 hours before microbiological analysis was completed. The paired *t*-test was used to compare initial and final pHs and the MF counts obtained initially with those from 2, 6, 12, 24, 30, and 48 hours. The MF and MPN counts were compared by using correlations and analysis of variance for repeated measures. All analyses were performed with statistical programs.††

Results

Effect of sample holding time and temperature on total coliform counts. Total coliform MF counts from selected samples of finished drinking water held at 5°C and

*Hach Kit CN-87, Hach Co., Ames, Iowa
†Model 2100A, Hach Co., Ames, Iowa
‡Model 31 Conductivity Bridge, Yellow Springs Instrument Co., Yellow Springs, Ohio
§Difco Co., Detroit, Mich.
**API 20E strips, Analytab Products Div., Ayerst Laboratory, Inc., Plainview, N.Y.
††Statistical Analysis System (SAS), SAS Inst., Inc., Cary, N.C.

22°C and analyzed initially and at 24, 30, and 48 hours are depicted in Figures 2 and 3. Initial counts ranged from 5/100 mL to 130/100 mL. Coliform densities in samples held at 22°C with initial counts of 31 or less/100 mL declined to levels of 4 or less/100 mL in all but one sample at 24 hours and in all samples at 30 hours. The degree of coliform decline varied widely among individual samples, but consistent trends were observed at each holding temperature. The rates of decline were much sharper at 22°C (Figure 4) than at 5°C. The line graph shows mean coliform counts, expressed as $\log_{10}(N_t/N_0)$, for all samples at each temperature. Coliform loss was 50 percent or more after 24 hours in 17 percent (2/12) of the samples held at 5°C and in all samples (12/12) held at 22°C. The graph illustrates the rapid decline in MF and MPN coliform counts at 22°C, in contrast to the decline at 5°C. The mean rate of decline was 2.5 orders of magnitude greater at 22°C than at 5°C.

Rates of decline for the two holding temperatures most closely followed a linear rather than curvilinear regression model, and the rates of decline for each of three methods of analysis—linear regression, MLE, and $\log_{10}(N_t/N_0)$—at a given temperature were similar (Table 1). Paired *t*-test results showed coliform losses for the two temperatures were significantly different at 24, 30, and 48 hours.

The greater variability of coliform rates of decline at 22°C is reflected in increased 95 percent confidence intervals for this group (Tables 2 and 3). These tables show that in 30 hours, coliform counts declined by 40–50 percent at 5°C and by 70–95 percent at 22°C.

Effects of environmental factors on initial total coliform counts. Initial counts of coliforms measured by the MF technique for 12 samples were significantly correlated (-0.7) at the 5 percent level with sample collection day and (0.68) initial temperatures (7°C to 25°C) but not with free chlorine (0.0 to 0.22 mg/L), pH (6.83 to 9.1), turbidity (0.2 to 30 ntu), or specific conductivity (166 to 530 μmhos/cm).

From August to December 1982, the variable factors examined followed certain trends. The pH and free chlorine increased, whereas initial coliform counts, initial temperature, and conductivity decreased. Initial coliform counts declined linearly and were significantly correlated negatively with sampling day and initial temperature. Relationships with other environmental factors were not as well defined.

Effects of environmental factors during the holding period on total coliform rates of decline. Regression and MLE analyses indicated collection days, as measured from the initiation of the study, and, to a lesser extent, pH, had a significant effect on rates of decline for samples held at 5°C (Table 4). Unlike the correlation analysis, neither of these methods showed initial

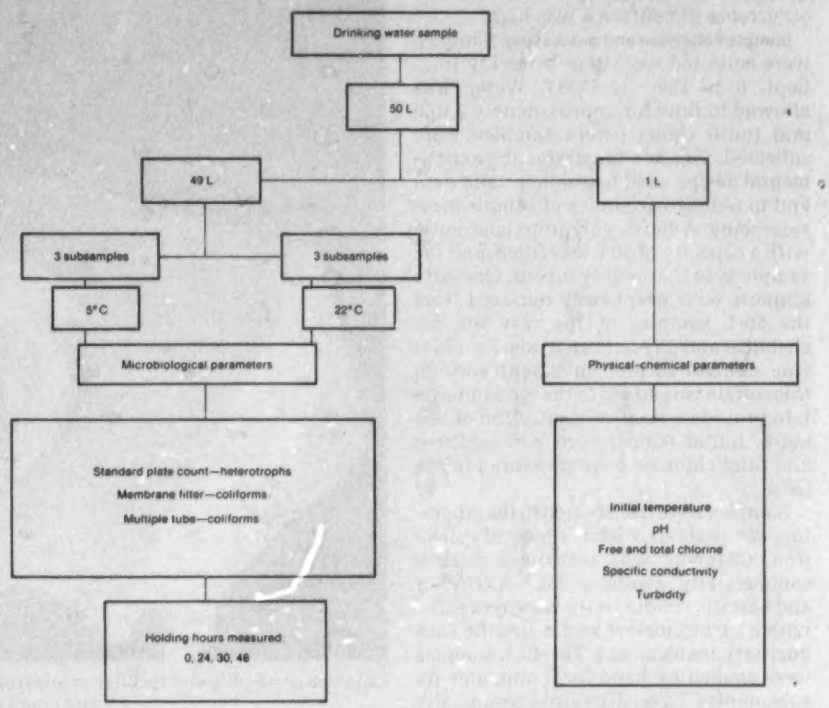


Figure 1. Schematic outline of procedures followed for sample processing

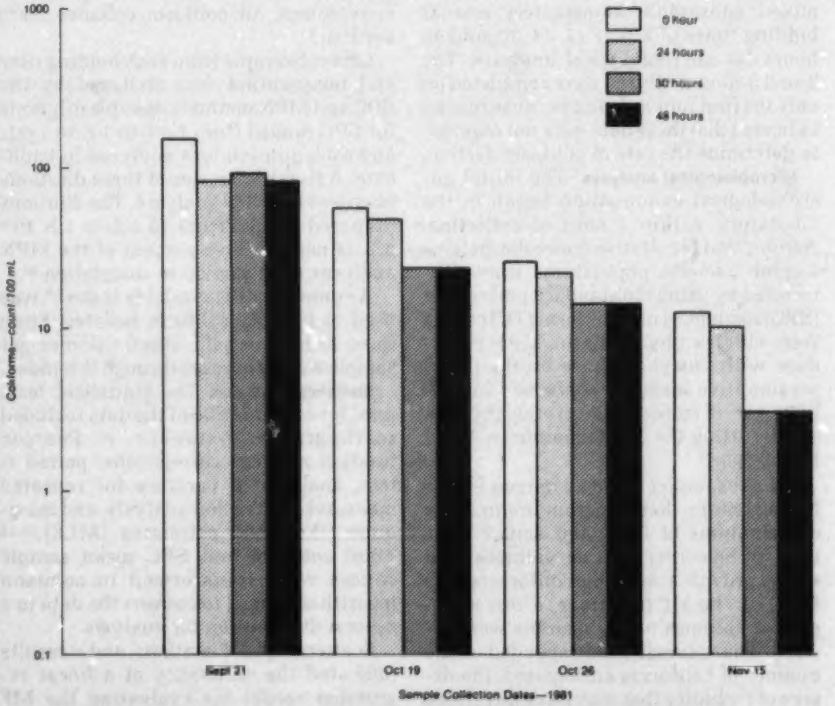


Figure 2. Coliform densities for selected drinking water samples held at 5°C

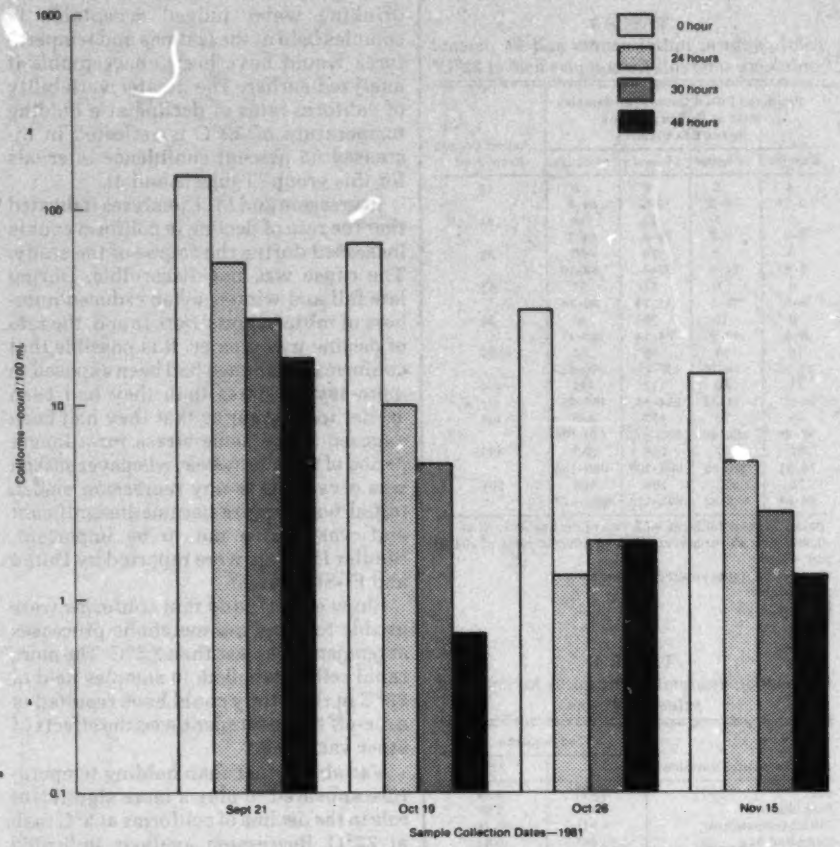


Figure 3. Coliform densities for selected drinking water samples held at 22°C

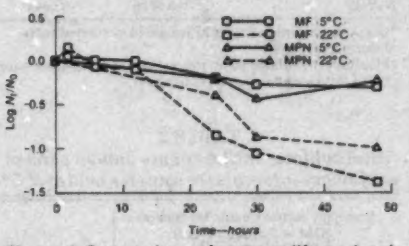


Figure 4. Comparison of mean coliform densities in drinking water samples held up to 48 hours

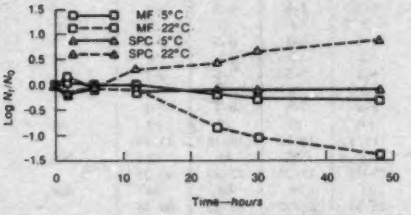
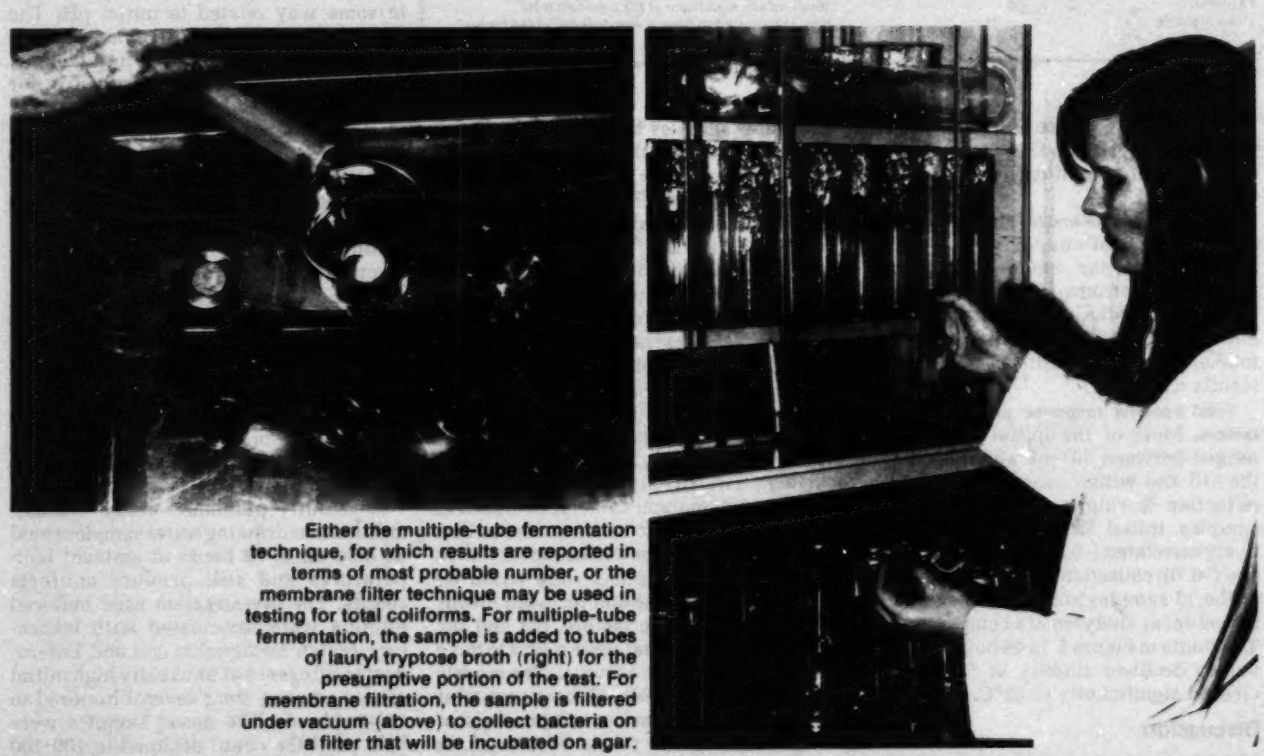


Figure 5. Comparison of mean coliform and heterotrophic bacteria densities in drinking water samples held up to 48 hours



Either the multiple-tube fermentation technique, for which results are reported in terms of most probable number, or the membrane filter technique may be used in testing for total coliforms. For multiple-tube fermentation, the sample is added to tubes of lauryl tryptose broth (right) for the presumptive portion of the test. For membrane filtration, the sample is filtered under vacuum (above) to collect bacteria on a filter that will be incubated on agar.

TABLE 1
A comparison of group mean rates of decline for coliform counts recovered by the MF technique

Rate of Decline Method	Temperature 5°C	Temperature 22°C
Linear regression*	-0.0076	-0.0349
MLE†	-0.0090	-0.0321
N_t/N_0‡	-0.0078	-0.0341

*Log_{10} mean sample values of weighted-predicted rates of decline per hour
†Maximum likelihood mean rate estimates of log_{10} per hour
‡Log_{10} N_t/N_0 mean recoveries per hour

TABLE 2
*Total coliform initial counts and 95 percent confidence intervals for samples held at 5°C**

Predicted Initial Counts for Samples Held at Different Hours Before Examination				Actual Counts Recovered
6 hours	12 hours	24 hours	30 hours	
1	1	2	2	1‡
1-1†	1-1	2-2	2-2	
2	3	3	4	2‡
2-2	3-3	4-3	4-3	
3	4	5	6	3‡
4-3	4-4	6-5	6-5	
5	5	7	8	4‡
5-4	5-5	7-6	9-7	
6	6	8	9	5§
6-6	7-6	9-8	11-8	
11	13	17	19	10§
12-11	14-12	18-15	21-17	
23	26	33	37	20§
23-22	27-25	37-30	43-33	
34	39	49	56	30§
35-33	41-37	55-45	64-50	

*Based on total coliform MLE rate of decline at 5°C of -0.00902 and 95 percent confidence interval rates of -0.011 and -0.0073
†Lower and upper confidence limits
‡Acceptable
§Unacceptable

TABLE 3
*Total coliform initial counts and 95 percent confidence intervals for samples held at 22°C**

Predicted Initial Counts for Samples Held at Different Hours Before Examination				Actual Counts Recovered
6 hours	12 hours	24 hours	30 hours	
2	2	6	9	1‡
2-1†	4-2	13-3	24-3	
3	5	12	18	2‡
4-3	7-3	25-5	48-7	
5	7	18	28	3‡
6-4	11-5	38-8	72-10	
6	8	24	37	4‡
8-5	14-5	51-11	96-14	
8	12	30	46	5§
9-6	16-8	64-14	120-17	
16	24	59	92	10§
19-13	36-16	127-27	240-35	
31	49	117	184	20§
38-27	71-33	254-54	480-69	
46	73	177	275	30§
57-38	106-49	381-81	720-104	
62	97	236	367	40§
76-51	142-66	508-108	960-138	
78	121	294	458	50§
94-64	178-82	635-135	2000-173	

*Based on total coliform MLE rate of decline at 22°C of -0.0321 and 95 percent confidence interval rates of -0.046 and -0.018
†Lower and upper confidence limits
‡Acceptable
§Unacceptable

TABLE 4
Regression analysis F-statistics for coliform rates of decline

Environmental Variables	Temperature 5°C	Temperature 22°C
pH	7.08*	0.14
Free chlorine	4.11	2.07
Initial temperature	6.71†	1.78
Sampling day	10.08*	0.81
Initial count	6.10†	0.16

*Statistically significant at the 5 percent level
†Insignificant when season was included in the model

counts or initial temperature to be important factors, because they became insignificant when sampling day was included in the model.

Comparison of MF and MPN sample coliform recoveries. The MF and MPN results closely paralleled one another (Figure 4). Analysis of variance for repetitive measurements by the two methods at three holding times (24, 30, and 48 hours) indicated that the counts were not significantly different.

Total bacteria response to environmental factors. Most of the initial SPC values ranged between 10^3/mL and 10^4/mL. As the fall and winter seasons advanced, a reduction in counts was found. For 20 samples, initial SPC values were negatively correlated (-0.85) with conductivity and (-0.70) collection day. The SPC values of the 12 samples statistically evaluated for coliform analyses are compared with TC counts in Figure 5. In 48 hours the SPC values declined slightly at 5°C but increased significantly at 22°C.

Discussion

Coliform populations in finished drinking water samples held at 5°C and 22°C declined significantly after 24, 30, and 48 hours but not after 2, 6, or 12 hours, as measured by the MF and MPN methods. Unacceptable initial MF counts of 5/100 mL and 17/100 mL for two samples held at 5°C for 24 hours declined to acceptable levels of 4 or less/100 mL, as did initial counts ranging from 5/100 mL to 31/100 mL for five samples held at 22°C. Mean coliform recovery at 5°C was 66, 54, and 54 percent for 24, 30, and 48 hours, respectively; at 22°C, the recovery was 13, 8, and 5 percent for 24, 30, and 48 hours, respectively. All of these losses were statistically significant.

The mathematical models developed in this study predicted coliform losses that closely matched the losses observed in these tests. The models were based on MLE rates of decline and included means of the two variable factors, pH and the day of collection, that were found to have an effect on coliform survival. The models indicated that initial coliform counts of 7/100 mL to 9/100 mL in samples held at 5°C and of 14/100 mL to 96/100 mL in samples held at 22°C could have declined to acceptable levels in 30 hours. Therefore, drinking water judged acceptable in samples held at these times and temperatures would have been unacceptable if analyzed earlier. The greater variability of coliform rates of decline at a holding temperature of 22°C is reflected in increased 95 percent confidence intervals for this group (Tables 2 and 3).

Regression and MLE analyses indicated that the rate of decline in coliform counts increased during the course of the study. The cause was not discernible. During late fall and winter, when reduced numbers of initial counts were found, the rate of decline was greater. It is possible that coliforms found then had been exposed to more severe stress than they had been earlier in the year or that they had been exposed to the same stress for a longer period of time. However, whenever season was a variable in any regression model, initial temperature became insignificant and was shown not to be important. Similar findings were reported by Dutka and El-Shaarawi.[8]

Shaw et al[13] found that coliforms were unable to carry out metabolic processes at temperatures less than 7.5°C. The more rapid cell metabolism in samples held at 22°C in this study could have resulted in a die-off that overshadowed the effects of other variables.

Variables other than holding temperature appeared to play a more significant role in the decline of coliforms at 5°C than at 22°C. Regression analysis indicated that rates of coliform die-off at 5°C were in some way related to initial pH. The mean initial pH for all samples was 7.9, and the maximum was 9.0. The higher pH values were found more frequently for water samples collected in the final days of the study when the rate of coliform decline was also greater, and the higher alkalinity could have been a factor in increased stress on coliforms. Differences between initial and final (48 hours) pH readings for each sample were not significant, as determined by the paired *t*-test.

Some workers have claimed interference in coliform recovery by SPC counts greater than 10^3/mL.[14] In this study, for samples held at 22°C for 30 hours, TC counts declined, SPC values increased, and confluent growth of noncoliforms on MF plates from large volumes (100 mL) frequently made coliform counts inaccurate or impossible.

A recently published study[15] recommended that drinking water samples could be held up to 72 hours at ambient temperatures and still produce coliform counts. The investigation used buffered dilution water inoculated with laboratory-grown *Escherichia coli* and *Enterobacter aerogenes* at unusually high initial counts ranging from several hundred to 2000/100 mL. The dosed samples were held until the count declined to 100/100 mL before effects of holding time and

temperature were measured. For some samples, confluent growth of noncoliforms on the plates was noted. However, the recommendation that drinking water samples can be held 72 hours prior to analysis cannot be considered valid because the samples used in the study did not reflect normal drinking water characteristics.

Conclusions

Results from this study indicated that because of coliform losses during holding, samples with counts that were initially unacceptable would have been reported as acceptable if held for 24 hours at either ambient or refrigerator temperatures. No significant changes were seen in samples held 6 hours. Therefore, it is recommended that samples be iced and analyzed within 6 hours of collection to prevent changes in coliform counts. Furthermore, it is recognized that results from this study, based on a drinking water supply system in one geographical area, may not be representative of public water supplies at other locations in the United States. To ensure that results from many types of water studied in different geographical areas of the country can be compared, it will be necessary for other investigators to use comparable experimental designs and statistical methods.

Acknowledgments

The authors are grateful for the assistance of J. Bryant of the University of Cincinnati's statistics department, who guided and performed much of the statistical analysis, and for the technical assistance of K.P. Brenner, J.R. Haines, D.W. O'Neill, and C.C. Rankin. The authors also thank D.B. Morris for typing the manuscript.

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